



# Variant in CD209 promoter is associated with severity of liver disease in chronic hepatitis C virus infection

Elizabeth J. Ryan <sup>a,\*</sup>, Megan Dring <sup>a</sup>, Cliona M. Ryan <sup>b</sup>, Carol McNulty <sup>c</sup>, Nigel J. Stevenson <sup>a</sup>, Matthew W. Lawless <sup>d</sup>, John Crowe <sup>d</sup>, Niamh Nolan <sup>b</sup>, John E. Hegarty <sup>b</sup>, Cliona O'Farrelly <sup>a</sup>

<sup>a</sup> School of Biochemistry and Immunology, Trinity College, Dublin, Ireland

<sup>b</sup> Department of Pathology, St. Vincent's University Hospital, Dublin, Ireland

<sup>c</sup> National Liver Transplantation Unit, St. Vincent's University Hospital, Dublin, Ireland

<sup>d</sup> Centre for Liver Disease, Mater Misericordiae University Hospital, Dublin, Ireland

## ARTICLE INFO

### Article history:

Received 15 February 2010

Accepted 5 May 2010

Available online 12 May 2010

### Keywords:

CD209

DC-SIGN

Hepatitis C virus

Chronic viral infection

Single-nucleotide polymorphism

## ABSTRACT

CD209, a c-type lectin expressed by dendritic cells (DCs), acts as a pathogen recognition receptor. A single nucleotide polymorphism (SNP) in the promoter region of *CD209* (–336 A/G; rs4804803) affects transcription and is associated with the severity of tuberculosis and dengue fever. Because CD209 binds hepatitis C virus (HCV) glycoprotein-E2, we investigated this SNP in the context of chronic HCV infection. A total of 131 Irish women who had received HCV-contaminated anti-D-immunoglobulin and 79 healthy control subjects were genotyped. We found no association between rs4804803 and the risk of HCV chronicity. However, of those with chronic infection, possession of at least one g-allele was associated with more advanced liver disease, with significantly higher liver fibrosis scores and levels of alanine transaminase (ALT) observed. We conclude that rs4804803, an SNP in the *CD209* promoter, contributes to severity of liver disease in chronic HCV infection.

© 2010 American Society for Histocompatibility and Immunogenetics. Published by Elsevier Inc. All rights reserved.

## 1. Introduction

Hepatitis C virus (HCV) infection is a major global public health problem [1]. Approximately 70% of acute HCV infections result in chronic infection with the subsequent potential to develop a wide spectrum of clinical sequelae, from asymptomatic chronic hepatitis to end-stage liver disease. The host's immune response to HCV is critical in determining whether resolution or persistent viremia will ensue after initial infection [2]. Viral impairment of dendritic cell (DC) function may be a major contributory factor to the establishment of chronic infection, though this remains controversial [3,4].

DCs use a wide variety of c-type lectin receptors to recognize pathogen-associated carbohydrate (CHO) structures. CD209 (DC-SIGN, DC-specific ICAM-3 grabbing non-integrin), the most widely studied molecule of this family, exhibits mannose-type CHO specificity and mediates the recognition of a plethora of diverse pathogens, including HCV, human immunodeficiency virus (HIV), dengue, *Helicobacter pylori*, and *Mycobacterium tuberculosis* by the innate immune system [5]. Capture of circulating HCV particles by CD209 expressed by DCs may facilitate virus infection of proximal hepatocytes and lymphocyte subpopulations and may be essential for the establishment of persistent infection [6]. However the pre-

cise role that this receptor plays in regulating the immune response to HCV infection or pathogenesis remains unclear.

A variant in the CD209 promoter region (rs4804803) that results in altered CD209 transcription is associated with the severity of dengue fever in a Thai population [7], tuberculosis in a sub-Saharan African population [8] and an increased risk of parental acquisition of HIV in an American–European population [9]. In this study, we investigated the association of rs4804803 with failure resolve HCV infection or the severity of liver disease in a cohort of Irish women who received HCV genotype 1b contaminated anti-D-immunoglobulin in 1977 and 1978.

## 2. Subjects and methods

### 2.1. Study subjects

A total of 131 who attend the hepatitis C clinic at St. Vincent's University Hospital (SVUH), Dublin, or the Mater Misericordiae Hospital, Dublin, were enrolled in this study. All were infected with HCV genotype 1b after receiving contaminated anti-D-immunoglobulin from a single source between May 1977 and November 1978 [10]. All patients consumed less than 14 alcohol units per week and had no other risk factors for liver disease. Informed written consent was obtained from each patient. The study received ethical approval from the Research and Ethics Committee at St. Vincent's University Hospital and conforms to the guidelines of the 1975 Declaration of Helsinki. All 131 patients tested positive for

\* Corresponding author.  
E-mail address: [eryan1@tcd.ie](mailto:eryan1@tcd.ie) (E.J. Ryan).

**Table 1**

Allelic and genotype frequencies of rs4804803 in a homogenous cohort of Irish patients who were exposed to HCV contaminated anti-D-immunoglobulin and healthy controls

|           | HCV RNA-positive |           | HCV RNA-negative |           | Healthy controls |           | Total    |           |
|-----------|------------------|-----------|------------------|-----------|------------------|-----------|----------|-----------|
|           | <i>n</i>         | Frequency | <i>n</i>         | Frequency | <i>n</i>         | Frequency | <i>n</i> | Frequency |
| <i>a</i>  | 118              | 0.75      | 80               | 0.77      | 128              | 0.81      | 326      | 0.78      |
| <i>g</i>  | 40               | 0.25      | 24               | 0.23      | 30               | 0.19      | 94       | 0.22      |
| Total     | 158              | 1         | 104              | 1         | 158              | 1         | 420      | 1         |
| <i>aa</i> | 45               | 0.57      | 32               | 0.62      | 55               | 0.70      | 132      | 0.63      |
| <i>ag</i> | 28               | 0.35      | 16               | 0.31      | 18               | 0.23      | 62       | 0.30      |
| <i>gg</i> | 6                | 0.08      | 4                | 0.07      | 6                | 0.07      | 16       | 0.08      |
| Total     | 79               | 1         | 52               | 1         | 79               | 1         | 210      | 1         |

No significant differences in genotype or allele frequency were observed between the groups (allele,  $p = 0.3945$ ; genotype,  $p = 0.5239$ ,  $\chi^2$  test).

antibodies to HCV using a third-generation enzyme immunoassay (Abbott Diagnostics, Wiesbaden, Germany), confirmed with an immunoblot assay (RIBA-3; Chiron Corp., Emeryville, CA). Of these patients, 79 (60%) remained chronically infected with the virus as determined by testing consistently positive for HCV RNA by a qualitative reverse transcriptase–polymerase chain reaction (RT-PCR; Amplicor; Roche Diagnostic Systems, Nutley, NJ), over a 15-year follow-up period. Of the patients, 52 (40%) achieved spontaneous (untreated) viral clearance and tested negative for HCV RNA by RT-PCR. None of the patients had received antiviral therapy at the time of entry to the study. Seventy-nine healthy volunteers were recruited from the general public. All of the patients completed a detailed health questionnaire, and none of the volunteers reported risk factors for viral hepatitis.

## 2.2. Assessment of liver disease

Alanine aminotransferase (ALT) data were available for all patients from medical records. At the time of diagnosis in 1994, percutaneous liver biopsies were performed on all HCV RNA-positive patients, with repeat biopsies performed on a subset of patients at irregular time intervals. Liver fibrosis scores were available for all patients in the study; fibrosis and inflammation was assessed by a single experienced pathologist in the SVUH patient cohort and included in our analysis. Fibrosis was classified according to the following scale: 0 = no fibrosis, 1 = periportal or portal fibrosis in a minority of tracts, 2 = portal fibrosis in most portal tracts, 3 = occasional portal-portal septae, 4 = occasional bridges, 5 = marked fibrosis with early nodule formation, and 6 = probable or definite cirrhosis. Inflammation was graded by light microscopy examination of the sections. The stage of fibrosis was assessed by Masson's trichrome staining and the Shikata orcein method. Inflammation was graded on a cumulative 18-point scale, with interface hepatitis graded from 0 to 4, confluent necrosis from 0 to 6, lobular inflammation from 0 to 4, and portal inflammation from 0 to 4.

## 2.3. Genomic DNA extraction and SNP genotyping

Genomic DNA was extracted from whole blood (HCV patients) using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, or from saliva swabs (controls) using a salting-out technique as described elsewhere [11]. Genomic DNA concentration was assessed using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA), and samples were diluted to 2 ng/ml in  $\text{dH}_2\text{O}$ . Genotyping of the CD209 variant rs4804803 was performed using a custom Taqman SNP Genotyping Assay (Applied Biosystems, Foster City, CA). The primer sequences used were as follows: 5'-ACTGTGTTACACCCCTCCAC-TAG-3' (sense), 5'-AGGAAAGCCAGGAGGTCACA-3' (antisense). The sequences of the Taqman probes were as follows: 5'-CTACCTGC-CCACCC-3' and 5'-CTGCCTACCTTGC-3'. The probes were labeled with the fluorescent dyes VIC and FAM, respectively. Polymerase chain reactions (PCRs) were set up according to the manufacturer's instructions. Thermal cycling was performed on a fast optical, 96-

well reaction plate on the 7500 Fast Real-Time PCR system (Applied Biosystems) as follows: initial denaturation and enzyme activation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minute. The genotype of each sample was attributed by measuring the allele-specific fluorescence on the 7500 Fast Real-Time PCR system (Applied Biosystems).

## 2.4. Statistical analysis

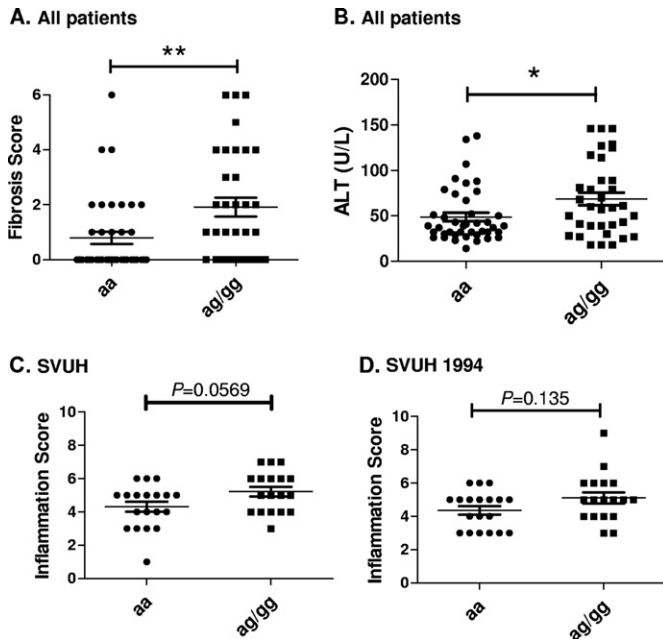
Allelic and genotypic frequencies were compared between the patient and control groups by the  $\chi^2$  test. Differences in ALT levels, liver fibrosis scores, and liver inflammation scores between patients of differing genotypes were determined using a two-tailed Mann-Whitney *U* test. In addition, we verified that fibrosis scores (0 vs >0) were associated with the SNP using Fisher's exact test on a  $2 \times 2$  contingency table of counts, with columns corresponding to genotype and rows to fibrosis scores (e.g., 0 vs >0). Values of  $p < 0.05$  were considered significant. All statistical analyses were performed using GraphPad Prism Version 5 (GraphPad Software, La Jolla, CA). Deviations from the Hardy-Weinberg equilibrium were calculated using a  $\chi^2$  Hardy-Weinberg equilibrium test calculator for biallelic markers [12].

## 3. Results

We investigated the association of the SNP rs4804803 in the promoter region of CD209 with the resolution of HCV infection and the progression of liver disease. Genomic DNA was obtained from healthy Irish volunteers ( $n = 79$ ), women with chronic HCV infection ( $n = 79$ ), and women who had resolved infection ( $n = 52$ ) having received HCV-contaminated anti-D-immunoglobulin and genotyped for the rs4804803 SNP. We found no difference in the frequency of the *a* or *g* alleles or *aa*, *ag*, and *gg* genotypes among healthy volunteers, women with chronic HCV infection (HCV RNA positive), or women who had spontaneously cleared the virus (HCV RNA negative) (Table 1).

Women in this cohort, in general, had relatively mild liver disease [10,13]; however some had progressed to develop liver fibrosis or cirrhosis. We wished to examine whether there was any difference in the progression of liver disease in women with the *aa* or *ag* and *gg* genotypes of the rs4804803 SNP in the CD209 promoter. Clinical data, including liver biopsy results, were available for 41 women for the *aa* genotype and for 34 women for the *ag* and *gg* genotype. Interestingly, patients with the *ag* or *gg* genotype had significantly worse liver fibrosis scores (Fig 1A), taking the most recently available fibrosis score of patients at both of the centers included in this study, and higher ALT levels (Fig 1B) than patients with the *aa* genotype. No significant differences in HCV viral loads were observed among the groups (median viral loads [range]: *aa*,  $2.532 \times 10^6$  IU/ml [ $0.29 \times 10^6$ – $4.94 \times 10^7$ ] and *ag/gg*,  $4.035 \times 10^6$  [ $1.257 \times 10^6$ – $2.36 \times 10^8$ ];  $p = 0.1519$ , Mann-Whitney *U* test).

The majority of the patients in this cohort have mild liver inflammation (<6, based on 18-point Histological Activity Index



**Fig. 1.** Comparison of clinical parameters of liver disease of HCV PCR<sup>+</sup> patients, with either the *aa* or *ag* genotype of rs4804803. Clinical data, including liver biopsy results were available for 41 patients with the *aa* genotype and 34 patients with the *ag* or *gg* genotype of rs4804803. Biopsy specimens were scored according to Ishak's grading and staging method that is the modified HAI system [23]. The most recent ALT results were obtained from medical records. (A) Most recent liver fibrosis scores (median year, 2002; range, 1994–2009). (B) 1994 fibrosis scores for SVUH patients. (C) ALT levels (U/L). (D) Liver biopsy liver inflammation scores were compared between the two groups using a two-tailed Mann–Whitney *U* test. \* *p* < 0.05 and \*\* *p* < 0.01. The central bar represents the mean and the error-bars the standard error of the mean.

[HAI] scale). We observed no significant difference between the inflammation scores of HCV RNA-positive women based either on recent biopsy data (Fig 1D) or on their 1994 baseline biopsy (Fig 1E). These data were from the SVUH cohort and were scored by a single experienced pathologist.

#### 4. Discussion

The underlying genetic reasons why some individuals resolve HCV infection and others fail to do so is still not fully understood. The Irish cohort is ideal for studying genetic associations related to HCV infection, as these individuals are infected with virus from a single source, and confounding factors are minimal. There is good evidence that certain human leukocyte antigen (HLA) class I and II alleles influence the outcome of HCV infection in this cohort of patients. HLA-A\*03, HLA-B\*27, and HLA-Cw\*01 occurred more frequently in individuals who cleared the virus [14], whereas the HLA-A\*11, Cw\*04 haplotype was associated with persistent infection [15]. The HLA class II allele DRB1\*01 was associated with spontaneous viral clearance [16]. Variation in genes encoding for other immune mediators, such as killer cell immunoglobulin-like receptors [17] and cytokines, e.g., IL-10, IL-22 [18] and IL-28 [19], have a role in determining an individual's response to HCV infection.

Previous work has demonstrated that CD209 has a crucial role in determining the outcome of infection with dengue virus, which, like HCV, is a member of the *Flaviviridae* family of viruses [7]. Among individuals with dengue, genotypes *gg* or *ag* strongly increase the risk of contracting dengue hemorrhagic fever versus dengue fever. Dengue hemorrhagic fever (DHF) is a potentially deadly complication that is characterized by high fever, often with enlargement of the liver, and in severe cases circulatory failure. In

our patient cohort, although liver inflammation was generally mild, there was a tendency toward higher inflammation scores (*p* = 0.0569) in patients with the *ag* or *gg* phenotype, which, along with higher levels of ALT levels and fibrosis scores, suggests that the possession of at least one *g* allele may increase the risk for liver disease progression. The SNP rs4804803 was demonstrated to be in linkage disequilibrium with three other intronic polymorphisms in the Thai population [7], and these may also contribute to the observed phenotype.

Interestingly, the allele frequency of rs4804803 in the healthy Irish control population deviated from Hardy–Weinberg equilibrium (HWE), with an overrepresentation of *aa* genotypes and a lower than expected frequency of *ag* genotypes observed. Higher prevalence of the *a* variant, which is associated with higher CD209 expression, in the healthy population may be associated with increased pathogen recognition and control of infection. For example, the *a* allele may confer protection against TB, resulting in an increased frequency in European populations compared with African populations because of a longer history of TB exposure [20].

Pathogen interaction with CD209 influences the subsequent antigen-specific immune response [21]. Mannose-dependent binding of pathogens such as *M. tuberculosis* and HIV-1 to CD209, leads to Raf-1-dependent upregulation of IL-10, IL-6, and IL-12 secretion [22]. In contrast, the fucose-dependent interaction of *H. pylori* or soluble *Schistosoma mansoni* egg antigen with CD209 induces IL-10 but suppresses IL-12, resulting in inhibition of T<sub>H</sub>1 responses. This difference in response is due to the effect of the ligand on the composition of the CD209 signalosome; effector proteins that activate Raf-1 are recruited in response to mannose-based ligands, whereas fucose-expressing pathogens actively dissociate from the KSR1–CNK–Raf-1 complex [22]. HCV glycoprotein E2 has 11 N-linked glycosylation sites; most oligosaccharides on E2 are high-mannose structures, suggesting that the interaction of E2 with CD209 would lead to the induction of both IL-10 and IL-12, as is the case with HIV-1 and *M. tuberculosis*. Therefore, we hypothesize that the DCs of individuals who possess the *g* allele of rs4804803 and thus lower levels of CD209 do not respond to E2 as efficiently as individuals who are homozygous for the *a* allele.

In conclusion, we have evidence that a variant in the promoter of CD209 is associated with worse liver disease in a well-defined cohort of patients who are homogenous for age, gender, race, mode of acquisition and HCV genotype. Overall, the risk of increased disease severity in HCV infection results from the complex multi-factorial virus–host interaction of which CD209 variability is 1 important factor.

#### Acknowledgments

We would like to thank all the patients who participated in this research study. We thank Catherine Keogh, Tanya Dempsey, and Avril Buckley for the expert technical assistance and patient database management, and Andrew Lloyd for helpful discussion. This study was supported by a Translational Research Award from the Irish Health Research Board.

#### References

- Thomas DL, Seeff LB. Natural history of hepatitis C. *Clin Liver Dis* 2005;9:383–98.
- Thimme R, Lohmann V, Weber F. A target on the move: Innate and adaptive immune escape strategies of hepatitis C virus. *Antivir Res* 2006;69:129–41.
- Szabo G, Dolganiuc A. Subversion of plasmacytoid and myeloid dendritic cell functions in chronic HCV infection. *Immunobiology* 2005;210:237–47.
- Decalf J, Fernandes S, Longman R, Ahloulay M, Audat F, Lefrerre F, et al. Plasmacytoid dendritic cells initiate a complex chemokine and cytokine network and are a viable drug target in chronic HCV patients. *J Exp Med* 2007;204:2423–37.
- van Vliet SJ, Garcia-Vallejo JJ, van Kooyk Y. Dendritic cells and C-type lectin receptors: Coupling innate to adaptive immune responses. *Immunol Cell Biol* 2008;86:580–7.

- [6] Cormier EG, Durso RJ, Tsamis F, Boussemart L, Manix C, Olson WC, et al. L-SIGN (CD209L) and DC-SIGN (CD209) mediate transinfection of liver cells by hepatitis C virus. *Proc Natl Acad Sci U S A* 2004;101:14067–72.
- [7] Sakuntabhai A, Turbpaiboon C, Casademont I, Chuansumrit A, Lowhnoo T, Kajaste-Rudnitski A, et al. A variant in the CD209 promoter is associated with severity of dengue disease. *Nat Genet* 2005;37:507–13.
- [8] Vannberg FO, Chapman SJ, Khor CC, Tosh K, Floyd S, Jackson-Sillah D, et al. CD209 genetic polymorphism and tuberculosis disease. *PLoS ONE* 2008;3:e1388.
- [9] Liu H, Carrington M, Wang C, Holte S, Lee J, Greene B, et al. Repeat-region polymorphisms in the gene for the dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin-related molecule: Effects on HIV-1 susceptibility. *J Infect Dis* 2006;193:698–702.
- [10] Kenny-Walsh E. Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin. Irish Hepatology Research Group. *N Engl J Med* 1999;340:1228–33.
- [11] Lahiri DK, Bye S, Nurnberger JI Jr, Hodes ME, Crisp M. A non-organic and non-enzymatic extraction method gives higher yields of genomic DNA from whole-blood samples than do nine other methods tested. *J Biochem Biophys Methods* 1992;25:193–205.
- [12] Rodriguez S, Gaunt TR, Day IN, Hardy-Weinberg equilibrium testing of biological ascertainment for Mendelian randomization studies. *Am J Epidemiol* 2009;169:505–14.
- [13] Nanda KS, Ryan EJ, Murray BF, Brady JJ, McKenna MJ, Nolan N, et al. Effect of chronic hepatitis C virus infection on bone disease in postmenopausal women. *Clin Gastroenterol Hepatol* 2009;7:894–9.
- [14] McKiernan SM, Hagan R, Curry M, McDonald GS, Kelly A, Nolan N, et al. Distinct MHC class I and II alleles are associated with hepatitis C viral clearance, originating from a single source. *J Hepatol* 2004;40:108–14.
- [15] Fanning LJ, Kenny-Walsh E, Shanahan F. Persistence of hepatitis C virus in a white population: Associations with human leukocyte antigen class 1. *Hum Immunol* 2004;65:745–51.
- [16] Barrett S, Ryan E, Crowe J. Association of the HLA-DRB1\*01 allele with spontaneous viral clearance in an Irish cohort infected with hepatitis C virus via contaminated anti-D immunoglobulin. *J Hepatol* 1999;30:979–83.
- [17] Khakoo SI, Thio CL, Martin MP, Brooks CR, Gao X, Astemborski J, et al. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. *Science* 2004;305:872–4.
- [18] Hennig BJ, Frodsham AJ, Hellier S, Knapp S, Yee LJ, Wright M, et al. Influence of IL-10RA and IL-22 polymorphisms on outcome of hepatitis C virus infection. *Liver Int* 2007;27:1134–43.
- [19] Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 2009;461:798–801.
- [20] Barreiro LB, Neyrolles O, Babb CL, Tailleux L, Quach H, McElreavey K, et al. Promoter variation in the DC-SIGN-encoding gene CD209 is associated with tuberculosis. *PLoS Med* 2006;3:e20.
- [21] den Dunnen J, Gringhuis SI, Geijtenbeek TB. Innate signaling by the C-type lectin DC-SIGN dictates immune responses. *Cancer Immunol Immunother* 2009;58:1149–57.
- [22] Gringhuis SI, den Dunnen J, Litjens M, van der Vlist M, Geijtenbeek TB. Carbohydrate-specific signaling through the DC-SIGN signalosome tailors immunity to Mycobacterium tuberculosis, HIV-1 and Helicobacter pylori. *Nat Immunol* 2009;10:1081–8.
- [23] Ishak K, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, et al. Histological grading and staging of chronic hepatitis. *J Hepatol* 1995;22:696–9.